Tao-Hong-Si-Wu Decoction promotes angiogenesis after cerebral ischaemia in rats via platelet microparticles

CHEN Fang-Fang1, 2Δ, WANG Meng-Meng1, 2Δ, XIA Wen-Wen1, 2, PENG Dai-Yin1, 2, 3, HAN Lan1, 2*

1 School of Pharmacy, Anhui University of Chinese Medicine, Hefei 230012, China; 2 Anhui Province Key Laboratory of Chinese Medicinal Formula, Hefei 230012, China; 3 Key Laboratory of Xin’an Medicine, Ministry of Education, Anhui University of Chinese Medicine, Hefei 230012, China

Available online 20 Aug., 2020

[ABSTRACT] Platelet microparticles (PMPs) are membrane particles derived from the platelet membrane that enter into the blood circulation. We sought to explore the therapeutic effects of Tao-Hong-Si-Wu Decoction (THSWD) on angiogenesis in a rat model of cerebral ischaemia-reperfusion (I/R). The protective effect of THSWD on I/R rats was observed morphologically by immunohistochemical expression of VEGF and CD34, along with immunofluorescence results of co-expression of BrdU and vWF. Then, PMPs from different groups of rats were extracted, and cytokine array analysis was used to screen for angiogenesis associated proteins. The results showed that THSWD can promote the expression of VEGF, CD34, BrdU and vWF. Cytokine array analysis revealed the changes in the expression of 29 related angiogenic proteins in the total protein of PMPs, which involved the Notch signalling pathway. Compared with model group, the expression levels of NICD and Hes-1 in the THSWD group were significantly increased. In the context of I/R, the angiogenesis-related proteins of PMPs are different. THSWD may involve the promotion of activation of the Notch signalling pathway to achieve therapeutic effects on cerebral ischaemia.

[KEY WORDS] Tao-Hong-Si-Wu Decoction; Platelet microparticles; Cerebral ischaemia–reperfusion; Angiogenesis

[CLC Number] R965

[Article ID] 2095-6975(2020)08-0620-08

Introduction

Ischaemic stroke is a leading cause of death worldwide [1]. Recent studies have found that therapeutic angiogenesis represents a new strategy [2]. As the most abundant microparticles in the blood of healthy individuals, platelet microparticles (PMPs, also called platelet microvesicles) are shed from the plasma membranes of platelets and have been associated with various physiological and pathological processes in vivo, including angiogenesis, cell-cell communication, and biomarkers, that are diagnosed in a variety of diseases [3]. Platelets may also release smaller microparticles called exosomes (40 to 100 nm in diameter). Many groups reported that PMPs contain large amounts of trophic factors and could function as early biomarkers in ischaemic disease. Proteomic analysis has demonstrated that 1179 proteins were identified from brain endothelial cells in the isolated extracellular microvesicles (EVs), which contain potential novel receptors for crossing the blood–brain barrier [4]. Circulating EVs include transport vehicles for large numbers of specific miRNAs involved in fundamental cellular processes related to ischaemic disorders. A sensitive detection assay was reported to assess early biomarkers of Alzheimer’s disease in exosomes [5].

In China, the usage of compound traditional Chinese medicine (TCM) preparations to treat cerebrovascular diseases dates back to the Han Dynasty. In the theory of Chinese Medicine, many cerebrovascular diseases were classified to syndrome of blood stasis, such as stroke [4]. Tao-Hong-Si-Wu Decoction (THSWD) originated from the Yizong Jinjian (Golden Mirror of Medicine, 1749) which was written by WU Qian during the Qing Dynast. It is composed of Rehmannia glutinosa Libosch., Angelica sinensis (Oliv.) Diels, Paeonia lactiflora Pall., Ligusticum chuanxiong Hort., Prunus persica (L.) Batsch and Carthamus tinctorius L.. It is a famous TCM formula for treating blood stasis syndrome for hundreds of years. The clinical application of THSWD is extremely extensive to treat a variety of diseases with blood stasis syndrome. Our previous studies have demonstrated that THSWD possesses potent protective in vascular dementia, and the mechanisms probably involved promoting...
cerebrovascular growth \(^7\).

Although research has supported the angiogenesis effect of THSWD in the I/R model, further study is needed to elucidate proteins that are regulated by THSWD. We proposed that the angiogenesis effect of THSWD may be accomplished by modulating various proteins in PMPs. In this study, we used cytokine array analysis to identify specific proteins in PMPs that mediate the angiogenesis effect of THSWD in the I/R model, and we validated the related signalling pathways according to the results of the cytokine arrays.

**Materials and Methods**

**Materials**

5-Bromo-2’-deoxyuridine (111D038) was purchased from Solarbio (Beijing, China). The Proteome Profiler Rat XL Cytokeine Array (ARY030) was purchased from R&D Systems (Minnetonka, USA). Anti-VEGF (E8562) was bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). These anti-body (vWF: GB11020, NICD: ZS-6014, 1 : 200) were purchased from ZSGB-BIO (Beijing, China), anti-body (CD34: AG07207545, Jagged1: bs-1448R, 1 : 1000; CCN1: bs-1290R, 1 : 300) were purchased from Bioss (Beijing, China), anti-body (Resistin: PAA847Ra01, 1 : 500; WISP-1: PAG895Mu01, 1 : 500) were purchased from USCN (Wuhan, China). Anti-Hes-1 antibody (ab108937, 1 : 1000) was bought from Abcam (Cambridge, MA, USA).

All animal experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Anhui Medical University Experimental Animal Center (Permit number: SCXK(jin)2016-0006). All of the experiments in the study were approved by the Institutional Ethical Committee of Anhui University of Chinese Medicine.

**Traditional Chinese herbs**

All herbal drugs were purchased from Puren Pharm. Co. (Anhui, China) and identified by Prof. Wang De-Qun (Anhui University of Chinese Medicine). Shi-Di-Huang (Rehmannia glutinosa) Libosch., No. 1705312, Dang Gui (Angelica sinensis) (Oliv.) Diels, No. 1611085), Bai-Shao (Paeonia lactiflora Pall., No. 17110114), Chuan-Xiong (Ligusticum chuanxiong) Hort., No. 17010335), Tao-Ren (Punus persica (L.) Batsch, No. 1702181) and Hong-Hua (Carthamus tinctorius L., No. 17072135) were mixed in a ratio of 4 : 3 : 3 : 2 : 3 : 2 and extracted with 75% ethanol for 2 h in a volume 10 times that of the total volume of the above drugs. The filtrate was retained, and the residue was extracted with 75% ethanol for 2 h. The amount of the filtrate was 8 times the volume of the total weight of the above drugs. The filtrate was collected twice and rotary evaporated, and finally concentrated to 1.8 g mL\(^{-1}\) and stored at 4 °C for later animal dosing.

**UPLC analysis of THSWD**

UPLC was performed on a Waters ACQUITY UPLC system (Waters, Milford, MA, USA). An ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) was used in the UHPLC system. THSWD was tested according to the routine procedure described in our previous report \(^8\).

**Middle cerebral artery occlusion-reperfusion (MCAO/R) model surgery and treatment**

Adult male Sprague-Dawley rats, weighing 280 to 320 g, were anaesthetized by intraperitoneal injection of pentobarbital (50 mg·kg\(^{-1}\)). Cerebral I/R injury was produced in SD rats by using the MCAO/R model and performed as previously described \(^9\). Briefly, this was accomplished by inserting a diameter of 0.285 mm silicon-coated nylon into the right external carotid artery and extending it to the beginning of the middle cerebral artery, nearly 18 to 20 mm from bifurcation to MCAO. After 2 h of occlusion, the filament was removed to allow MCAO reperfusion. Rats in the control group underwent the same surgical procedures without insertion of the filament. Throughout the procedure, the body temperature was maintained at 37 °C with a heating pad.

Rats were divided randomly into 6 groups by testing as Zea Longa described: control, model, THSWD (4.5, 9, and 18 g·kg\(^{-1}\)), and nimodipine group (20 mg·kg\(^{-1}\)), and gavage lasted for 7 days. Thereafter, the blood was used for PMPs preparation, cytokine array analysis and western blot.

**Detection of angiogenesis status by Immunohistochemistry staining and Immunofluorescence staining**

To determine the angiogenesis in rats, we evaluated the expression levels of VEGF (1 : 200) and CD34 (1 : 200) using immunohistochemistry staining. To determine the neonatal endothelial cells by immunofluorescence staining of BrdU, vWF and DAPI. BrdU was used to label recently proliferated cells. 5-Bromo-2’-deoxyuridine (50 mg·kg\(^{-1}\) in saline) was injected intraperitoneally at 8 : 00, 10 : 00, 12 : 00 and 14 : 00 on day 5 after I/R. The brains were removed at 14 : 00 on day 7. Sections were incubated with primary antibodies (BrdU, 1 : 100, vWF, 1 : 300) and then with secondary antibodies labelled with fluorescent dyes (goat anti-mouse, 1 : 300, ZSGB-BIO, Beijing, China, GB21301, goat anti-rabbit, 1 : 400, ZSGB-BIO, Beijing, China, GB25303). Finally, nuclei were stained with DAPI (C1005, Beyotime Biotechnology) for 5 min at room temperature. All images were captured using an immunofluorescence microscope (NIKON ECLIPSE TI-SR, Tokyo, Japan). Imaging analysis software (ImageJ, USA) was used to quantify fluorescence intensity by a researcher blinded to treatments.

**PMPs preparation**

As described in the literature \(^10, 11\), PMPs were prepared by differential centrifugation. Blood samples were collected in ACD anticoagulant with 5 : 1 ratio. Platelet-rich plasma (PRP) was obtained after 20 min of centrifugation at 200 g. Platelets from PRP were washed twice in PBS containing 4.2 mmol·L\(^{-1}\) ethylenediaminetetraacetic acid (EDTA) and 1 mmol·L\(^{-1}\) alprostadil to avoid platelet aggregation. After centrifugation at 800 g for 15 min, the pellet of platelets was resuspended in freshly filtered Tyrode’s solution with 5 mmol·L\(^{-1}\) CaCl\(_2\) and stimulated with thrombin for 30 min at room temperature. For PMPs isolation, the supernatant was further centrifuged for 60 min at 100 000 g at 4 °C in a Beckman Counter OptimaTM L-90k Ultracentrifuge (75004380,
Thermo Fisher Scientific, Germany). After the ultracentrifugation, the supernatant was carefully removed and the remaining PMPs enriched suspension was resuspended in filtered PBS for labelling and analysis.

Flow cytometric analysis

Flow cytometry was performed by Cytoflex Flow cytometer (Beckman, USA). First, latex beads (Sigma, L4655, USA) with a diameter of 1 μm were used to adjust appropriately for detection of PMPs. For determination of PMPs surface markers, PMPs were labelled at 4 °C for 30 min with anti-Mouse/Rat CD61 APC (1 : 200, BioLegend, USA), and APC Armenian Hamster IgG Isotype Ctrl (1 : 200, Biolegend, USA) was used as a negative control. PMPs were identified as membrane vesicles smaller than 1 μm with CD61¹.

Transmission Electron Microscopy (TEM) and nanoparticle tracking analysis (NTA)

The sample (10 μL) was dropped onto a copper grid and precipitated for 3 min. Phosphotungstic acid was added for negative staining. After drying at room temperature for 2 min, samples were imaged on a computer (JEM-1200EX, Japan; voltage of the electron microscope ranging from 80 to 120 kV). NTA was performed using a Nanosight LM10 (Nanosight/Malvern, Worcestershire, UK) to determine the PMPs size distributions with a 1 mL sample.

Cytokine array

Cytokine array analysis in platelet microparticles was performed using the Proteome Profiler Rat XL Cytokine Array from R&D Systems on the basis of the manufacturer’s instructions.

Western blot

RIPA lysates were added directly to the PMPs suspension in a 1 : 4 ratio. Ultrasonic crushing operations were performed 5 times on ice for 5 seconds each time. After standing for 30 min, the loading buffer was added in a 1 : 4 ratio and then boiled for 8 min. Western blotting was performed as described previously.

Statistical analysis

The data are expressed as the means ± standard deviation (SD) from at least three independent experiments. One-way analysis of variance (ANOVA) was used to compare the differences between groups. In all cases, P < 0.05 was considered statistically significant.

Results

UPLC profile of THSWD

The major components of THSWD were analysed by UPLC (Fig. 1). Compared with the standard reference compound, six compounds were identified and determined, namely, hydroxysafflor yellow A, amygdalin, paeoniflorin, ferulic acid, verbascoside and ligustilide. Their respective contents in THSWD were identified as 0.198, 0.45, 0.602, 0.031, 0.014 and 0.256 mg mL⁻¹.

THSWD attenuated infarction volume in I/R model

As shown in Fig. 2, compared to control rats, model group increase in the infarction volume. THSWD and nimodipine treatment improve the infarction volume signific-
The Rat XL Cytokine Array Kit was used according to the instructions. As shown in Fig. 6, the results showed that the fold changes of 29 proteins were above 1.1. Most of these proteins have a direct or indirect role in promoting angiogenesis. Based on the fold change value, five related differentially expressed proteins were selected for verification. These proteins were Resistin, WISP-1/CCN4, Cyr61/CCN1, Jagged1 and MMP-9, representing different levels of difference change values. According to the results of western blotting (Figs. 6B and 6C), the THSWD group exhibited significantly increased expression of five proteins compared with the model group. Moreover, the trend of protein changes is the same as that of the cytokine profiling array, although the fold change is different.

**THSWD enhanced the activity of the Notch signalling pathway**

To investigate the relationships among I/R, THSWD and Notch signalling pathway, we detected the expression levels of the Notch intracellular domain (NICD) and Hes-1. As shown in the western blot results (Fig. 7), MCAO/R increased the expression of NICD by 1.41-fold, and Hes-1 by 1.64-fold. Moreover, after treatment with THSWD, the levels...
of NICD further increased by 3.35-fold, 2.19-fold and 1.43-fold, and the level of Hes-1 further increased by 2.83-fold, 1.91-fold and 1.5-fold, respectively, compared with the MCAO/R groups. These results demonstrated that MCAO/R

Fig. 4 Effects of THSWD on the expression of angiogenesis in the penumbra of rats (400 ×). (A) Immunofluorescence graph. (B) Quantification of BrdU/vWF fluorescence signal. The results were presented as the means ± SD (n = 3). **P < 0.01 vs the control group; ***P < 0.01 vs the model group, tested by one-way ANOVA and the Fisher’s PLSD

Fig. 5 Characteristics and identification of PMPs. (A) Representative histogram for CD61⁺ PMPs, PMPs blank: Blank control group, PMPs: Sample group, PMPs ISO: Isotype control group. (B) Electron microscope image of PMPs. (C) Results of nanoparticle tracking analysis of PMPs
activated the Notch signalling pathway and that THSWD strengthened the activity in a dose-dependent manner.

Discussion

Angiogenesis is activated after stroke, and it is widely accepted that angiogenesis after ischaemic stroke ameliorates the restoration of blood supply in the ischaemic zone [12]. I/R increases infarct volume and neuronal cell damage. Angiogenic factors are generally involved in the process of angiogenesis in the brain. We found that on day 7, the treat-
ment group showed a significant increase in VEGF and CD34 expression compared to the I/R group. VEGF protein expression was observed to increase gradually between 2 and 14 days after MCAO, and there was evidence of new angiogenesis during days 7–28 [13]. Transmembrane glycoprotein CD34 regulates cell migration during neovascularization [14]. BrdU was used to label recently proliferated cells. vWF was released by endothelial cells and megakaryocytes. Therefore, vWF was considered to be a marker of endothelial damage. We found that the expression levels of BrdU and vWF in brain tissue were higher in the treatment group (Fig. 4). These indicated that THSWD could promote the proliferation of newly born cells and increased vascular endothelial cells after ischaemic injury. Our results demonstrated that THSWD increased neurogenesis and angiogenesis (Figs. 3 and 4). Flow cytometry is the most commonly used method for detecting PMPs. PMPs are commonly labelled with monoclonal antibodies to CD41, CD42a and CD61, but this technique is limited by a lack of adequate standardization. The International Society for Extracellular Vesicles (ISEV) has recently reaffirmed the minimum experimental requirements for EVs research, including isolation, identification, and functional studies. Transmission electron microscopy combined with nanoparticle tracking analysis technology has become a common method for identifying individual vesicles in EVs [15]. Therefore, after extracting PMPs, we used flow cytometry, TEM and NTA methods to identify PMPs. Since monoclonal antibodies against rat CD41 and CD42a are not currently available, only CD61 expression experiments were performed herein. It has been reported that flow cytometry of PMPs in rats is performed only with CD61 [16].

Emerging evidence suggests that MPs are key messengers in intercellular communication, allowing cells to exchange proteins, lipids and genetic material [17]. Clinical applications of MPs for diagnosis, prognosis and treatment are being investigated [18]. The interaction between platelets and endothelial cells is crucial in the dysfunction and pathogenesis of cerebrovascular disease. Platelets are always one of the first cell types to reach the site when vascular dysfunction occurs in the body. It has been found that platelet aggregation in the brain microvessels of ischaemic mice is associated with endothelial activation in vivo [19]. As the most abundant MPs in the circulation, PMPs contain bioactive proteins and genetic materials from their parental cell. It has now been demonstrated that PMPs exert physiological effects on almost all blood and vascular cell types [20]. PMPs have been associated with angiogenesis, and this may be explained by the abilities of PMPs to enhance vascular permeability and to promote inflammation [21]. Platelet exosomes may have the potential to excite signals at a distance from the platelet activation site [22]. Our results indicated that protein levels associated with angiogenesis in PMPs were significantly changed after cerebral ischaemia. Even more so, the results also demonstrated that the improvement in THSWD after cerebral ischaemia can be reflected by the type and amount of proteins in PMPs. These proteins primarily include growth factors, matrix metalloproteinases, chemokines (CXCLs and CCLs) and interleukins. Given that PMP expresses most of the platelet membrane proteins, PMPs may act together with platelets to regulate the circulatory system in specific health and disease states associated with platelet activation.

As a complex process, angiogenesis includes matrix degradation, migration and proliferation of endothelial cells, as well as increasing vascular permeability [23]. More growth factors, matricellular proteins, chemokines, matrix metalloproteins and membrane proteins have been associated with angiogenesis in vivo. We screened 29 differential proteins and performed string analysis (https://string-db.org/). In our study, the fold changes of several proteins were most pronounced, including Resistin, WISP-1/CCN4, Cyr61/CCN1, MMP-9 and Jagged1, whereas PMPs did not affect VEGF-A or PDGF levels significantly. These changes of PMPs as revealed in the present study are similar to those reported by Sun et al. [23]. WISP-1/CCN4 is an extracellular matrix-related protein that regulates angiogenesis, and tumour-secreted WISP-1 increased angiogenesis-related tumour growth [24]. It is also described that CCN1 promotes an increase in endothelial progenitor angiogenesis in rheumatoid arthritis diseases [25]. Part of the mechanisms by which they function is due to the promotion of intracellular VEGF expression. Local injection of loaded (RANTES)/CCL5 microparticles in a mouse hindlimb ischaemia model can improve muscle regeneration and revascularisation [26].

In human brain endothelial cells of the peri-infarct region, the expression of the activated form of Jagged1 was increased [27]. String analysis (https://string-db.org/) of the differential proteins showed that the Jagged1 protein played an important role. The mammalian Notch signalling pathway involves five cognate ligands (DLL1, DLL3, DLL4, Jagged1 and Jagged2) and is important for angiogenesis. Notch signalling is dependent on the trans-binding of the Notch receptor to a ligand on an adjacent cell. Ligand binding promotes intracellular cleavage of the receptor by metalloproteinases to release the active form of NICD [28]. It is of interest that Jagged1 was the sole Notch ligand packaged into mesenchymal stem cell-derived exosomes [29]. Endocytosis was the predominant route of entry of PMPs into endothelial cells [30]. Activation of Notch1 signalling increases subventricular zone neurogenesis, while inhibition of Notch1 signalling reduces neurogenesis [31]. Notch signalling is a core event of chronic inflammation involved in the pathogenesis of cerebrovascular disease [32], so the interleukin-related factors in the factor antibody array analysis also exhibit significant changes. All of these findings support Notch activation as a favourable signal in I/R injury. In the present study, the expression of NICD and Hes-1 dramatically decreased in the I/R group. However, treatment of THSWD after I/R significantly promoted NICD and Hes-1 upregulation. These results implied that, in the context of I/R, THSWD improved ischaemic injury by targeting the NICD/Hes-1 signalling pathway. The shortcoming of this study is lack of in vitro evidence to demonstrate the role of PMPs in angiogenesis. More evidence should be used to demonstrate that THSWD promotes angiogenesis through PMPs and Notch signaling pathways.
Conclusion
The current study concludes that THSWD excellent angiogenesis and neuroprotective effects against I/R injury, including promoting PMPs to carry angiogenesis-related signaling molecules and activation of the Notch signalling pathway. These findings indicated a promising therapeutic potential for THSWD as a drug towards the treatment of ischemic stroke. Combining above data indicates that PMPs may be effective and potential biomarkers for the diagnosis and treatment of clinical cerebral ischaemia diseases.

References